REMARKS

Claim 1 has been amended. Support for the amendment to claim 1 may be found at, *inter alia*, page 12, lines 19-23, 26-27 and 21-32. Claims 1 and 18-25 remain pending in the application. Applicants submit that the amendment to claim 1 raises no issues of new matter and is fully supported by the specification as filed. Applicants respectfully request that this Amendment be entered.

Objections to the Specification

The Examiner stated on page 2, paragraph 1, of the August 4, 2009 Office Action that the abstract of the disclosure is objected to because it does not appear on a separate sheet. In response, applicants have amended the abstract of the disclosure so that it now appears on a separate sheet as required by 37 CFR 1.72(b). Accordingly, reconsideration and withdrawal of this ground of rejection is respectfully requested.

The Examiner stated on page 3, paragraph 2, of the August 4, 2009 Office Action that the specification of the instant application lacks the required format for presentation as provided in 37 CFR 1.77(b) as it does not include sections under different titles such as "Cross-reference to related

applications." In response, applicants have amended the specification to include a section heading entitled Cross-reference to the related applications and accompanying text as required under 37 CFR 1.77(b). Accordingly, reconsideration and withdrawal of this ground of rejection is respectfully requested.

The Examiner stated on page 3, paragraph 3, of the August 4, 2009 Office Action that the specification comprises several tables and none of the tables displays a number associated with the table to identify the table in the specification. In response, applicants have amended the specification to include a table number following each table.

Claim Objections

Claim 1 was objected to because claim 1 recites "—" in defining the limitations of "a) and b)" in the claim. In response, applicants have amended claim 1 to remove "--" from the claim. Applicants respectfully request withdrawal of the Examiner's objection.

Claim Rejections - 35 U.S.C. §112 Second Paragraph

Claims 1 and 18-25 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Specifically, the Examiner alleges on page 6, paragraph 1 that the recitation "glycocyamine (GA), equivalents thereof, and mixtures thereof includes ester and/or ether compounds without identifying the type of such compounds that would have the desired properties as defined and as required by the instant claims such that it is unclear from the claim as recited and from the disclosure as disclosed what the definition of "equivalents thereof" is since the chemical or structural characteristics of the compounds themselves are not adequately defined. In response, applicants respectfully traverse the Examiner's rejection. However, in order to expedite prosecution and without conceding the correctness of the Examiner's position, applicants have hereinabove amended claim 11 to recite, in relevant part, an energy metabolism precursor selected from glycocyamine (GA), guanidine-aceitic acid salts of sodium, potassium, calcium, ammonium, magnesium, zinc, iron, copper, chromium and mixtures thereof and/or GA bidentate with zinc to a maximum of 100 mg zinc per daily dose of the total composition; esters of acetic acid, propionic acid, butyric acid, and mixtures thereof; and

compounds where the amidino group of the GA is modified by protonation and thus forms a salt, as stated in the specification (page 12, lines 19-23, 26-27 and 31-32). Applicants respectfully request withdrawal of the Examiner's objection.

The Examiner also rejected claims 1 and 18-25 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention on page 7, paragraph 2. Specifically, the Examiner alleges that the recitation in claim 1 that "the composition is free of glycine, or if glycine is present in the composition, the weight ratio L-Serine to Glycine is more than 2.7:1 as determined by hydrolysis" is unclear as it does not identify the peptide or protein by name, the primary structural aspects of proteins or peptides in terms of SEQ ID NOs., or the source of such proteins or peptides that would have this desired requirement that the ratio of L-Serine to Glycine is more than 2.7:1 when Glycine is present in the composition. The Examiner also stated that it is unclear how the ratio of L-Serine:Glycine can be maintained at more than 2.7:1 in the composition when the hydrolysis of glycocyamine yields glycine sine the glycine present in the protein fraction and the GA upon

hydrolysis yields more glycine and hence the initial ratio of L-Serine:Glycine prior to hydrolysis would be vastly different from the ratio upon hydrolysis of the same composition due to the hydrolysis of GA to glycine. In response, applicants respectfully traverse the Examiner's rejection. Applicants note that the claim need not identify the peptide or protein by name as one of skill in the art would readily understand how to obtain a composition that would have the desired ratio of more than 2.7:1 of L-Serine to Glycine when Glycine is present in the composition. Applicants note that one of skill in the art would find sufficient guidance in the AOAC Official methods of Analysis 1984, nr 43.263 and 43.264 as referred to at page 11, lines 13-15 of the specification. By using the method described in the AOAC one of skill in the art would be able to determine the ratio upon hydrolysis and could thus verify whether or not a composition falls within the scope of the claims. Additionally, applicants note that the claim states that the ratio of L-serine to glycine is measured *after* hydrolysis of the composition by performing hydrolysis of the final product or mixture. Accordingly, applicants maintain that the ratio of L-Serine to Glycine *prior to* hydrolysis is irrelevant. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

The Examiner also rejected claim 18 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention on page 8, paragraph 3. Specifically, the Examiner alleges that the recitation in claim 18 of the "molar amount" is unclear because it is not clear how a relation between weight ratio of one variable, i.e., L-Serine:Glycine is used in the determination of molar amount of another variable, i.e. GA when the weight of Glycine in the composition itself would change upon hydrolysis of GA as illustrated on page 7, paragraph 2 of the August 4, 2009 Office Action. In response, applicants respectfully traverse the Examiner's rejection. As discussed above, applicants note that claim 1 states that the ratio of L-serine to glycine is measured *after* hydrolysis of the composition by performing hydrolysis of the final product or mixture. Accordingly, applicants maintain that the ratio of L-Serine to Glycine *prior to* hydrolysis is irrelevant. Applicants note that the excess of L-Serine versus glycine is determined in terms of weight of serine after hydrolysis (Specification page 11, lines 1-7). This weight value can easily be recalculated in terms of the molar amount of serine. The range of the energy

metabolism precursor recited in claim 18 can therefore be calculated by multiplying the molar amount of serine calculated above by 0.1 and 10. Applicants maintain that claim 18 is therefore not indefinite. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

The Examiner also rejected claim 18 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention on page 9, paragraph 4 of the August 4, 2009 Office Action. Specifically, the Examiner alleges that the amendment to claims 1 and 18 in applicants' June 23, 2009 response is not clear because applicants used single brackets to indicate that the text within the brackets has been deleted instead of using double brackets. In response, applicants note that the text within the single brackets was deleted. Applicants have not included the text contained within the brackets in the listing of claims above as the Examiner's analysis of the claims suggests that the Examiner treated the claims as if the text within the brackets was deleted. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

Claim Rejections - 35 U.S.C. §112, First Paragraph

Claims 1 and 18 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement because the claims allegedly contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner stated that the claims do not sate the name, source or structure of the protein or peptides that are the source of L-Serine amino acid that would have the desired requirement that the ratio of L-Serine to Glycine is more than 2.7:1 when Glycine is present in the composition. Additionally, the Examiner alleged that as the hydrolysis of GA yields gylcine, it is unclear how the ratio of L-Serine:Glycine can be maintained at more than 2.7:1. The Examiner also alleges that the initial ratio of L-Serine:Glycine prior to hydrolysis in the composition would be vastly different from the ratio upon hydrolysis of the same composition due to the hydrolysis of GA to gylcine. The Examiner also states that the ratio of L-Serine to Glycine in milk protein differs in each of several proteins. In response, applicants respectfully traverse the Examiner's rejection. As discussed above, applicants note that the claim need not identify the peptide

or protein by name as one of skill in the art would readily understand how to obtain a composition that would have the desired ratio of more than 2.7:1 of L-Serine to Glycine when Glycine is present in the composition. Applicants note that one of skill in the art would find sufficient guidance in the AOAC Official methods of Analysis 1984, nr 43.263 and 43.264 as referred to at page 11, lines 13-15 of the specification. By using the method described in the AOAC one of skill in the art would be able to determine the ratio upon hydrolysis and could thus verify whether or not a composition falls within the scope of the claims. Additionally, applicants note that the claim states that the ratio of L-serine to glycine is measured *after* hydrolysis of the composition by performing hydrolysis of the final product or mixture. Accordingly, applicants maintain that the ratio of L-Serine to Glycine *prior to* hydrolysis is irrelevant. Applicants also note that the claims do not require the use of milk protein as a source of L-Serine and Glycine. Additionally, applicants note that the information available from Swaisgood provides examples of protein sources wherein the ratio of L-Serine:Glycine is more than 2.7:1. Applicants maintain that such information would provide sufficient guidance to one of skill in the art to allow them to select an appropriate source protein such that

the ratio of L-Serine:Glycine is more than 2.7:1. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

The Examiner also rejected claim 18 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement because the claims allegedly contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, on page 12, second paragraph of the August 4, 2009 Office Action, the Examiner alleges that the recitation in claim 18 of the "molar amount" is unclear because it is not clear how a relation between weight ratio of one variable, i.e., L-Serine:Glycine is used in the determination of molar amount of another variable, i.e. GA when the weight of Glycine in the composition itself would change upon hydrolysis of GA as illustrated on page 7, paragraph 2 of the August 4, 2009 Office Action. In response, applicants respectfully traverse the Examiner's rejection. As discussed above, applicants note that claim 1 states that the ratio of L-serine to glycine is measured *after* hydrolysis of the composition by performing

hydrolysis of the final product or mixture. Accordingly, applicants maintain that the ratio of L-Serine to Glycine *prior to* hydrolysis is irrelevant. Applicants note that the excess of L-Serine versus glycine is determined in terms of weight of serine after hydrolysis (Specification page 11, lines 1-7). This weight value can easily be recalculated in terms of the molar amount of serine. The range of the energy metabolism precursor recited in claim 18 can therefore be calculated by multiplying the molar amount of serine calculated above by 0.1 and 10. Applicants maintain that claim 18 is therefore not indefinite. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

The Examiner also stated that the instant specification lacks a specific example wherein the final mixture of composition was subjected to hydrolysis and the weight ratio of L-Serine:glysine was shown to be more than 2.7:1. In response, applicants respectfully traverse the Examiner's rejection. Applicants note that the specification includes many examples of compounds that contain L-Serine in a ratio of more than 2.7:1 as compared to Glycine as these example compounds do not contain glycine. Accordingly, applicants respectfully request withdrawal of the Examiner's objection.

Claim Rejections - 35 U.S.C. §102

The Examiner rejected claims 1 and 18 under 35 U.S.C. § 102(b) as being unpatentable over McCoy, 1956, American journal of Veterinary Research, 17, 90-97 (hereinafter "McCoy"). The Examiner asserts that this reference teaches all of the claim elements of these claims.

Applicants maintain that claim 1 as amended and 18 are patentable over McCoy. The Examiner alleges that McCoy discloses a composition administered to dogs that comprised casein (and therefore L-Serine) combined with glycocyamine, which mixture is free of *free* glycine, and thus inherently falls within the scope of the claims. Applicants note that the claim does not mention "free glycine" at all but rather requires that the composition be either i) free of *any* glycine, including any glycine that may be in the amino acid content of proteins and peptides present in the composition, or, ii) if glycine is present, that the composition satisfies the requirement of a minimum L-Serine:glycine weight raito of 2.7:1. Contrary to the Examiner's allegation, applicants note that the composition of McCoy contains casein and thus contains glycine. The Examiner's own statement on page 7 of the August 4, 2009 Office Action states that "almost all protein from the natural source is made

up of naturally occurring amino acids and hence invariably contains glycine as one of the amino acids." The specification of the present application also indicates that "proteins always contain serine and glycine" (page 7, lines 12-13). Accordingly, applicants note that the mixture disclosed in McCoy *does* contain glycine and therefore does not fall within catergory (i) above. Applicants note that on its face, McCoy does not indicate whether or not it falls within category (ii) above, i.e. while McCoy contains L-Serine and Glycine, it does not indicate whether or not the ratio of L-Serine:Glycine is more than 2.7:1. Nonetheless, applicants maintain that the composition of McCoy does not contain a ratio of L-Serine:Glycine that is more than 2.7:1. Applicants attach hereto as **Exhibit** 1 a handbook authored by H.G. Kessler which indicates in Table 23.12 that casein contains L-Serine:Glycine in a ratio of 2.6:1, which is lower than the minimum requirement of 2.7:1 recited in claim 1. Accordingly, the features of the present invention as recited in amended claim 1 are not found in the teachings of McCoy. Applicants submit that independent claim 1 defines patentable subject matter over McCoy. Claims 18-25 depend from claim 1 and are also submitted to define patentable subject matter at least for the reasons set forth above. Reconsideration and withdrawal of these rejections is respectfully requested.

The Examiner rejected claims 1, 18 and 23 under 35 U.S.C. § 102(e) as being anticipated by Haik (US 6,727,285). The Examiner asserts that this reference teaches all of the claim elements of these claims.

Applicants maintain that claim 1 as amended and 18 are patentable over McCoy. The Examiner alleges that Haik discloses a composition of bovine serum albumin (BSA) a protein, glycocyamine (which reads on claims 1 and 18), and methylglyoxal (an aldehyde) (which reads on claim 23) in a composition buffered with HEPES. The Examiner alleges that this mixture is free of *free* glycine, and thus inherently falls within the scope of the claims. As discussed above, applicants note that the claim does not mention "free glycine" at all but rather requires that the composition be either i) free of *any* glycine, including any glycine that may be in the amino acid content of proteins and peptides present in the composition, or, ii) if glycine is present, that the composition satisfies the requirement of a minimum L-Serine:glycine weight raito of 2.7:1. Contrary to the Examiner's allegation, applicants note that the composition of Haik contains BSA and thus contains glycine, as indicated in Interaction Of SWP With Bovine Serum Albumin (BSA), available at http://www.friedli.com/research/PhD/chapter5a.html (a copy of

which is attached hereto as **Exhibit 2**). Accordingly, applicants note that the mixture disclosed in Haik *does* contain glycine and therefore does not fall within catergory (i) above. Moreover, as indicated in Exhibit 2, the weight ratio of L-Serine:Glycine in BSA is about 2.6. Accordingly, the composition disclosed in Haik does not contain a ratio of L-Serine:Glycine that is more than 2.7:1. Accordingly, the features of the present invention as recited in amended claim 1 are not found in the teachings of Haik. Applicants submit that independent claim 1 defines patentable subject matter over Haik. Claims 18-25 depend from claim 1 and are also submitted to define patentable subject matter at least for the reasons set forth above. Reconsideration and withdrawal of these rejections is respectfully requested.

Claim Rejection - 35 U.S.C. §103

The Examiner rejected claims 1 and 18-25 under 35 U.S.C. § 103(a) as being unpatentable over McCoy in view of Hageman, WO 99/03365 (hereinafter Hageman).

Applicants maintain that claim 1 as amended and claims 18-25 are patentable over McCoy in view of Hageman. The Examiner alleges that McCoy discloses a nutritional composition of low casein, supplemented with methionine and glycocyamine which

reads on the instant claim 1 as the composition of McCoy is free of *free* glycine. The Examiner also asserts that Hageman discloses a nutritional composition comprising casein as the protein as well as the components recited in claims 18-25, wherein the composition is free of *free* glycine. As discussed above, applicants note that claim 1 does not mention "free glycine" at all but rather requires that the composition be either i) free of *any* glycine, including any glycine that may be in the amino acid content of proteins and peptides present in the composition, or, ii) if glycine is present, that the composition satisfies the requirement of a minimum L-Serine:glycine weight ratio of 2.7:1. Contrary to the Examiner's allegations, applicants note that the compositions of McCoy and Hageman contain casein and thus contain glycine. Accordingly, applicants note that the mixtures disclosed in McCoy and Hageman *do* contain glycine and therefore do not fall within category (i) above. Applicants further note that, as discussed above, and as evidenced by Exhibit 1, casein contains L-Serine:Glycine in a ration of 2.6:1, which is lower than the minimum requirement of 2.7:1 recited in claim 1. Accordingly, the features of the present invention as recited in amended claim 1 are not found in the teachings of McCoy or Hageman, either alone or in combination. Applicants submit that

independent claim 1 defines patentable subject matter over McCoy in view of Hageman. Claims 18-25 depend from claim 1 and are also submitted to define patentable subject matter at least for the reasons set forth above. Reconsideration and withdrawal of this rejection is respectfully requested.

Nonstatutory Obviousness-Type Double Patenting

The Examiner rejected claims 1, 19 and 21-24 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. 6,544,547 ("the '547 Patent") in view of McCoy.

Applicants note that the '547 Patent is a national stage entry under 371(c)(1) of PCT application PCT/NL98/00408, which published as Hageman WO 99/03365. Accordingly, the specification of the '547 Patent and the Hageman publication cited by the Examiner in relation to the rejection under 103(a) are the same. As discussed above, neither the '547 Patent nor McCoy teach a composition that either is free of glycine or contains L-Serine:Glycine in a ration of more than 2.7:1. Accordingly, the features of the present invention as recited in amended claim 1 are not found in the teachings of McCoy or the '547 Patent, either alone or in combination. Applicants submit that independent claim 1 defines patentable

subject matter over McCoy in view of the '547 Patent. Claims 18-25 depend from claim 1 and are also submitted to define patentable subject matter at least for the reasons set forth above. Reconsideration and withdrawal of this rejection is respectfully requested.

In summary, Applicants submit that they have addressed and overcome all of the objections and rejections stated in the Office Action, and that the application now is in condition for allowance. Applicants request notice to this effect at the Examiner's earliest convenience.

Applicants, through the undersigned attorney, hereby petition the Commissioner of Patents to extend the time for responding to the Office Action dated August 4, 2009 for three months from November 4, 2009 to February 4, 2010.

Submitted herewith is a check for $1,110.00 to cover the cost of this extension.

No fee other than the $1,110 extension of time fee is believed to be required in connection with the filing of this Communication. However, the Commissioner is hereby authorized

to charge any fees required in connection with the filing of this Communication to Deposit Account No. 03-3125.

Respectfully submitted,

Dated: February 4, 2010

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Gregory J. Carbo 2/4/10
Gregory J. Carbo Date

Lebensmittel- und Bioverfahrenstechnik

Molkereitechnologie

H.G. Kessler

Vierte überarbeitete und erweiterte Auflage
mit 883 Abbildungen
und 109 Tabellen

ISBN 3-9802378-4-2

Verlag A. Kessler • D-80687 München

Tab. 23.12. Zusammensetzung (%) der Muttermilch und der Milch verschiedener Tierarten [BELITZ/GROSCH, 1987]

[a] Während der Stillperiode ab dem 15. Tag Anstieg auf 1,6 % Protein.

Art	Protein	Casein	Molkenprotein	Zucker	Fett	Asche
Mensch	0,9[a]	0,4	0,5	7,1	4,5	0,2
Esel	2,0	1,0	1,0	7,4	1,4	0,5
Pferd	2,5	1,3	1,2	6,2	1,9	0,5
Kamel	3,6	2,7	0,9	5,0	4,0	0,8
Rentier	10,1	8,6	1,5	2,8	18,0	1,5
Kuh	3,2	2,6	0,6	4,6	3,9	0,7
Zebu	3,2	2,6	0,6	4,7	4,7	0,7
Yak	5,8			4,6	6,5	0,9
Büffel	3,8	3,2	0,6	4,8	7,4	0,8
Ziege	3,2	2,6	0,6	4,3	4,5	0,8
Schaf	4,6	3,9	0,7	4,8	7,2	0,9
Katze	7,0	3,8	3,2	4,8	4,8	0,6
Hund	7,4	4,8	2,6			
Kaninchen	10,4					

Tab. 23.13. Aminosäurezusammensetzung (g AS/100g Protein) von Gesamtprotein, Casein und Molkenprotein der Kuhmilch [BELITZ/GROSCH, 1987].

Aminosäure	Gesamtprotein	Casein	Molkenprotein
Alanin	3,7	3,1	5,5
Arginin	3,6	4,1	3,3
Asparaginsäure	8,2	7,0	11,0
Cystin	0,8	0,3	3,0
Glutaminsäure	22,8	23,4	15,5
Glycin	2,2	2,1	3,5
Histidin	2,8	3,0	2,4
Isoleucin	6,2	5,7	7,0
Leucin	10,4	10,5	11,8
Lysin	8,3	8,2	9,6
Methionin	2,9	3,0	2,4
Phenylalanin	5,3	5,1	4,2
Prolin	10,2	12,0	4,4
Serin	5,8	5,5	5,5
Threonin	4,8	4,4	8,5
Tryptophan	1,5	1,5	2,1
Tyrosin	5,4	6,1	4,2
Valin	6,8	7,0	7,5

Tab. 23.14. Wichtige Eigenschaften der vier Caseinkomponenten [SCHMIDT, 1980; WALSTRA und JENNESS, 1984; FOX, 1989]

Caseinfraktion	α_{s1}	α_{s2}	β	κ
Molekulargewicht (Dalton)	23600	25200	24000	19000
IEP	4,1-4,8	5,1	4,8-5,1	5,5-5,8
Phosphatreste	8-9	10-13	5	1-2
Bindungskräfte für Micellenbildung	H-Brücken hydrophob	elektrostatisch	hydrophob	nicht bekannt
Calciumsensitivität	++	+++	+	-
Nettoladung bei pH 6,6	-21	-16 bis -22	-12	-4

Tab. 23.15. Physikalisch-chemische Charakteristika der Molkenproteinfraktionen [WALSTRA und JENNESS, 1984; KINSELLA und WHITEHEAD, 1989]

Fraktion	β-Lg	α-La	BSA	IG
Molekulargewicht (10^3 Dalton)	18,6	14,2	66,0	150-960
Konzentration in Milch [g/kg]	3,2	1,2	0,4	≈ 0,7
pH_{IEP} [-]	5,3	4,8	5,1	5,5-6,8
Cystingruppen [-]	2	4	17	32

Tab. 23.16. Zusammensetzung des Gesamtfettes der Kuhmilch (Gew. %) [SCHLIMME/BUCHHEIM, 1995]

Bestandteil	
Monoglyceride	0,02 - 0,10
Diglyceride	0,3 - 1,6
Triglyceride	96 - 99
Phospholipide	0,2 - 1,0
Cerebroside	0,01 - 0,07
Squalen	Spuren
Steroide	0,2 - 0,4
Wachse	Spuren
Freie Fettsäuren	0,1 - 0,4

Tab. 23.17. Fettsäurenzusammensetzung von Butterfett [SCHLIMME/BUCHHEIM, 1995]

		Mittelwert (Gew. %)
4:0	Buttersäure	4,0
6:0	Capronsäure	2,4
8:0	Caprylsäure	1,3
10:0	Caprinsäure	2,9
12:0	Laurinsäure	3,6
14:0	Myristinsäure	11,2
16:0	Palmitinsäure	28,2
18:0	Stearinsäure	9,4
18:1	Octadecensäure	23,5
18:2	Octadecadiensäure	2,1
18:3	Octadecatriensäure	1,7

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D-85350 Freising-Weihenstephan

Printed in Germany 1996

ISBN 3-9802378-4-2

kessler - lebensmittel- und bioverfahrenstechnik - molkereitechnologie

D-85350 Freising-Weihenstephan

Verlag A.Kessler, Agnes-Bernauer-Str. 174, D-80687 München

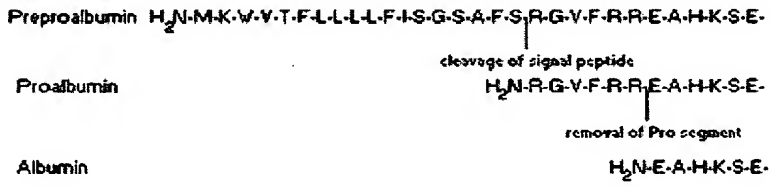
INTERACTION OF SWP WITH BOVINE SERUM ALBUMIN (BSA)

Introduction

Serum albumin is one of the most widely studied proteins and is the most abundant protein in plasma with a typical concentration of 5g/100ml. Various researchers have studied the structure and properties of serum albumin and its interaction with other proteins in order to understand how serum albumin affects the functionality of foods in which they have been included as well as novel applications. The latter reason led to the study of the interaction between soluble wheat protein and bovine serum albumin. The structure and properties of SWP are described in chapters 2, 3 and 4. The following sections describe the structure and properties of BSA.

Bovine serum albumin (BSA)

Albumin is generally regarded to mean serum albumin or plasma albumin (care should be taken to distinguish albumen, which refers to egg white, from albumin or serum albumin). The word albumin is also used to describe a protein or a group of proteins defined by solubility in water for example the albumin fraction of wheat (chapter 2). Albumin is the most abundant protein in the circulatory system and contributes 80% to colloid osmotic blood pressure (Carter and Ho, 1994). It has now been determined that serum albumin is chiefly responsible for the maintenance of blood pH (Figge *et al.*, 1991). In mammals albumin is synthesized initially as preproalbumin by the liver. After removal of the signal peptide, the resultant proalbumin is further processed by removal of the six-residue propeptide from the new N-terminus. The albumin released into circulation possesses a half-life of 19 days (Waldmann, 1977).



Structure of BSA

The substantial information on serum albumin has led to some contradictory results and discussions. Based largely on hydrodynamic experiments (Hughes, 1954; Squire *et al.*, 1968; Wright and Thompson, 1975) and low-angle X-ray scattering (Bloomfield, 1966), serum albumin was postulated to be an oblate ellipsoid with dimensions of 140 × 40Å (Figure 5.1). Experiments have continued to support these dimensions (Bendedouch and Chen, 1983; Feng *et al.* , 1988). Brown and Shockley (1982), compiled a diverse variety of data and constructed a model of albumin as having the shape of a cigar.

Figure 5.1: Classical preception of the structure of serum albumin (Peters, 1985).

However, studies using 1H NMR indicated that an oblate elipsoid structure of albumin was unlikely; rather a heart-shaped structure was proposed (Bos *et al.*, 1989). This was in agreement with X-ray crystallographic data (Carter *et al* ., 1989). Previous studies indicated that the secondary structure contained about 68% - 50% alpha-helix and 16% -18% beta-sheet (Sjoholm and Ljungstedt, 1973; Reed *et al.*, 1975; Foster, 1977). In contrast according to X-ray crystallography, there is no beta-sheet in the structure of native serum albumin (Carter *et al.*, 1989). Riley and Arndt (1952, 1953) suggested that thermally denatured bovine serum albumin has probably the same fundamental type of folding of the polypeptide chains as the native one, which is 55% alpha-helix and 45% random conformation from X-

ray scattering studies. Harmsen and Braam (1969), using infra-red and ORD spectra concluded that alkali or heat denaturation caused a partial loss of alpha-helical structure with no formation of beta-sheet but from their infra-red spectra, a shoulder appeared for BSA heated above 72°C at 1620 cm^{-1} indicative of beta-sheet formation. Clark *et al.* (1981b) testing Astbury's theory (Astbury *et al.*, 1935), that gels formed from heat or chemically denatured proteins arose from the interaction of regions of beta-sheets, carried out tests on BSA using infra-red and Raman spectra. A shoulder was visible on their infra-red spectra at 1620 cm^{-1} for BSA heated to 75°C and above. The Raman spectra showed an increase at 1235 cm^{-1} and a decrease at 940 cm^{-1} for the BSA gel, indicating a drop in alpha-helix content with formation of beta-sheet. Lin and Koenig (1976) investigated heat, acid and alkali denaturation of BSA by Raman spectroscopy and found that heating to 70°C or a change in pH to below 5 or above 10 caused an increase of the 1246 cm^{-1} band and a decrease of the 938 cm^{-1} band. The interpretation was similar to those of Clark *et al.*, (1981b) that is a decrease in the alpha-helix content accompanied by an increase in beta-sheet.

Amino acid composition

Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and the charged amino acids, aspartic and glutamic acids, lysine, and arginine. The glycine and isoleucine content of BSA are lower than in the average protein (Peters, 1985) (Table 5.1).

Table 5.1: Amino acid composition of BSA (Brown, 1975; Patterson and Geller, 1977; McGillivray *et al.*, 1979; Reed *et al.*, 1980; Hirayama *et al.*, 1990).

Ala 48	Cys 35	Asp 41	Glu 58
Phe 30	Gly 17	His 16	Ile 15
Lys 60	Leu 65	Met 5	Asn 14
Pro 28	Gln 21	Arg 26	Ser 32
Thr 34	Val 38	Trp 3	Tyr 21

Primary, secondary and tertiary structure

Figure 5.2 shows the bovine albumin amino acid sequence. The BSA molecule is made up of three homologous domains (I, II, III) which are divided into nine loops (L1-L9) by 17 disulphide bonds. The loops in each domain are made up of a sequence of large-small-large loops forming a triplet. Each domain in turn is the product of two subdomains (IA, IB, etc.). The primary structure of albumin is unusual among extracellular proteins in possessing a single sulfhydryl (Cys-34) group. In the light of new information i.e., x-ray crystallographic data (Carter and Ho, 1994) albumin structure is predominantly alpha-helical (67%) with the remaining polypeptide occurring in turns and extended or flexible regions between subdomains with no beta-sheets (Figure 5.3). Each of the domains can be divided into 10 helical segments, 1 - 6 for subdomain A and 7 - 10 for subdomain B (Figure 5.4). The motif for subdomain A is shown in Figure 5.5 and for subdomain B in Figure 5.6. Domains I and II and domains II and III are connected through helical extensions of 10 (I) - 1 (II) and 10 (II) - 1 (III), creating the two longest helices in albumin.

Disulphide bonds

In BSA the disulphide bonds are located in the following positions:

(1) 77-86; (2) 99-115; (3) 114-125; (4) 147-192; (5) 191-200; (6) 223-269; (7) 268-276; (8) 288-302; (9) 301-312; (10) 339-384; (11) 383-392; (12) 415-461; (13) 460-471; (14) 484-500; (15) 499-510; (16) 537-582; (17) 581-590.

The conformations of the disulphides are primarily gauche-gauche-gauche and $C\beta_1$-S1-S2-$C\beta_2$, with torsion angles clustering around ± 80°. The disulphide pairings are located almost exclusively between helical segments.

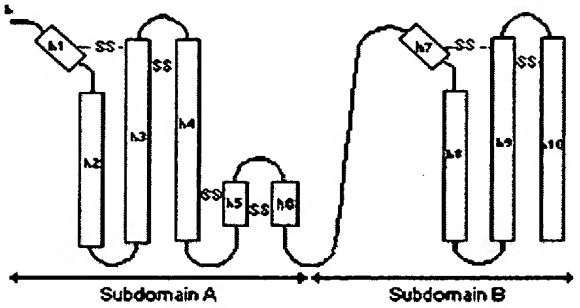


Figure 5.7: Location of disulphide bonds (He and Carter, 1992)

None of the disulphide bonds was accessible to reducing agents in the pH range 5-7, but became progressively available as the pH was raised or lowered. Katchalski *et al.*, (1957), therefore, concluded that the disulphides in albumin were protected at neutral pH from reducing agents. This is also apparent in the structure, which shows that the majority of disulphides are well protected and most are not readily accessible to solvent. Blocking of the free sulphydryl, Cys-34, with iodoacetamide, cysteine, or glutathione prevents the occurrence of mixed disulphides in aged albumin, as well as the occurrence of the albumin dimer (Peters, 1985). During unfolding, the conformation of some of the disulphide bonds change from the gauche-gauche-gauche to gauche-gauche-trans forms, as observed by laser Raman studies (Aoki *et al.*, 1982).

Physical-Chemical Properties

The albumin molecule is not uniformly charged within the primary structure. At neutral pH, Peters (1985) calculated a net charge of -10, -8, and 0 for domains I, II, and III for bovine serum albumin. The surface charge distribution is shown in Figure 5.8.

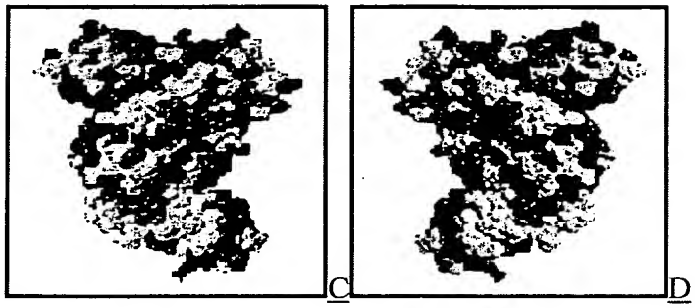


Figure 5.8: Space filling model of serum albumin molecule with basic residues coloured in blue, acidic residues in red, and neutral ones in yellow. (A) Front view, (B) back view, (C) left side, and (D) right side (Carter and Ho, 1994).

Unlike the asymmetric charge distribution on the primary structure, the distribution on the tertiary structure seems fairly uniform.

Viscosity

The viscosity of a protein solution depends on its intrinsic characteristics, such as molecular mass, size, volume, shape, surface charge and ease of deformation (Bull, 1940; Yang, 1961). In addition, viscosity is influenced by environmental factors such as pH, temperature, ionic strength, ion type, shear conditions and heat treatment (Tung, 1978; Lee and Rha, 1979; Hermansson, 1979b). Serum albumin has been reported to have intrinsic viscosity values of 3.7-4.2 ml g-1 (Peters, 1985; Kuntz and Kauzmann, 1974; Markus *et al.*, 1964; Reynolds *et al.*, 1967). Kolthoff *et al.*, (1958) reported an increase in viscosity with increased cleavage of the disulphide bonds of BSA. The viscosity of solutions of BSA increased linearly with concentration up to 65 mg ml-1 and exponentially at higher concentrations (Wetzel *et al.*, 1980) consistent with the results of Menjivar and Rha, (1980).

Effects of pH

Serum albumin undergoes reversible conformational isomerization with changes in pH.

```
                        E <--------> F <--------> N <--------> B <--------> A

pH of transition:              2.7           4.3           8            10
Name:                   Expanded      Fast          Normal        Basic        Aged
% Helix:        35          45             55            48           48
```

Figure 5.9: Relationship of isomeric forms of bovine serum albumin (Foster, 1977).

The N-F transition involves the unfolding of domain III (Geisow and Beaven, 1977; Khan, 1986). The F form is characterized by a dramatic increase in viscosity, much lower solubility, and a significant loss in helical content (Foster, 1960). At pH values lower than 4, albumin undergoes another expansion with a loss of the intra-domain helices (10) of domain I which is connected to helix (1) of domain II, and that of helix (10) of domain II connected to helix (1) of domain III (Figure 5.10). This expanded form is known as the (E) form which has an increased intrinsic viscosity, and a rise in the hydrodynamic axial ratio from about 4 to 9 (Harrington *et al.*, 1956). At pH 9, albumin changes conformation to the basic form (B). If solutions of albumin are maintained at pH 9 and low ionic strength at 3°C for 3 to 4 days, another isomerization occurs which is known as the (A) form.

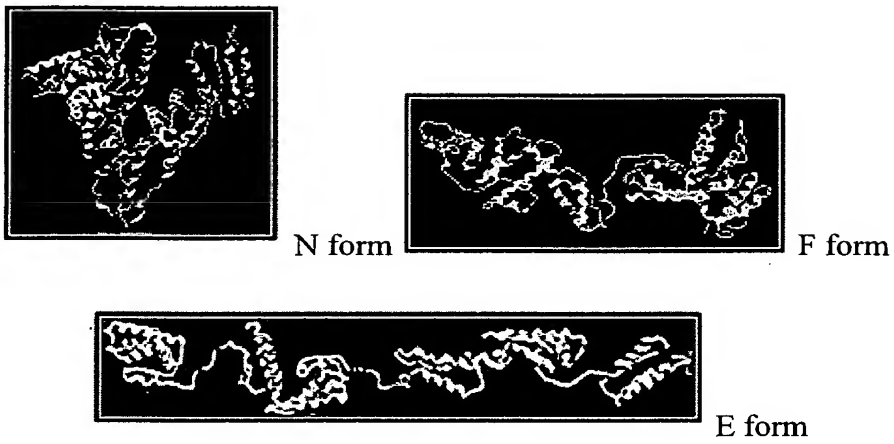


Figure 5.10: Ribbon diagram of serum albumin in its N form, and in its proposed F and E forms (Carter and Ho, 1994)

Effect of Heat

Serum albumin when heat-treated, goes through two structural stages. The first stage is reversible whilst the second stage is irreversible but does not necessarily result in a complete destruction of the ordered structure (Kuznetsow *et al.*, 1975; Lin and Koenig, 1976; Oakes, 1976). Heating up to 65°C can be regarded as the first stage, with subsequent heating above that as the second stage (Wetzel *et al.*, 1980). The onset temperature of conformation change as found by DSC was 58.1°C (Poole *et al.*, 1987) and the temperature of denaturation 62°C (Ruegg *et al.*, 1977). Results from CD and IR spectroscopy indicated that beta-sheets were formed when albumin was heated above 65°C (Wetzel *et al.*, 1980), 70°C (Lin and Koenig, 1976; Clark *et al.*, 1981b). The beta-sheet formed was more pronounced on cooling and was concentration dependent. Wetzel *et al.*, (1980) found a shoulder in the beta-sheet band at a concentration of 50mg/ml at 70°C, 1.4mg/ml at 80°C but not for 0.5mg/ml at 75°C. Because beta-sheet structures were not indicated in the dilute solution this suggests that the beta-sheets are intermolecular.

The sedimentation coefficient of albumin at neutral pH is around 4 - 4.5 S. If albumin samples are heated to more than 60°C and cooled down again, apart from the native protein, a faster sedimenting but heterogenous fraction with sedimentation coefficients of 26 - 36 S are found. The formation of these high molecular weight complexes was temperature and concentration dependent (Wetzel *et al.*, 1980). Spin labelling Cys-34 (Hull *et al.*, 1975; Wetzel *et al.*, 1980) and fluorescence investigations proved Cys-34 to be located in a pocket. X-ray crystallography indicated that Cys-34 is located in a crevice on the surface of the protein and that the reactive sulphur is protected by several residues (Figure 5.11). Cys-34 has been found to be the most reactive sulfhydryl with a pKSH of 5 compared with 8.5 and 8.9 for cysteine and glutathione (Pedersen and Jacobsen, 1980). Blocking of Cys-34 with cysteine, glutathione, or other chemicals such as N-iodosuccinimide stabilised albumin against dimer formation (Peters, 1985). Electron spin resonance measurements indicated that the pocket around Cys-34 unfolded during thermal denaturation (Wetzel et al., 1980). Therefore, dimer formation during heating is probably due to disulphide bonding.

Thus, it may be concluded that, in the reversible structural stage, some of the alpha-helices are transformed to random coils. If the side chains of neighbouring residues of two peptide strands point in opposite directions and are perpendicular to a plane so that hydrogen bonds can form between the the strands, then IR, CD, and Raman spectroscopy will see them as beta-sheets. This means that aggregates are formed through the hydrogen bonding of beta-sheets between monomers. The beta-sheets formed are

most likely to be antiparallel, since they are bound to be on the surface of the monomers. Parallel sheets are less twisted than antiparallel and are always buried. Antiparallel sheets can withstand greater distortions (twisting and beta-bulges) and greater exposure to solvent. As the temperature is increased past the reversible stage, unfolding of the pocket exposing Cys-34 takes place giving easy access to the formation of disulphide bridges. Since disulphide bridges are covalent bonds, this stage is irreversible.

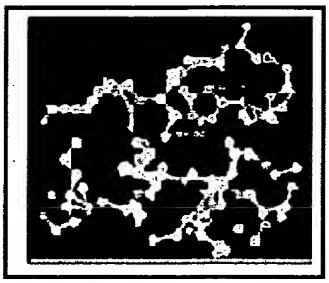


Figure 5.11: Stereo ball-stick model of serum albumin structure at the region around residue Cys-34. Red, oxygen; yellow, carbon; blue, nitrogen; green, sulphur. (Carter and Ho, 1994)

Richardson and Ross-Murphy (1981) put forward a hypothesis from their model of two intersecting lines, that the two processes, unfolding and aggregation can be distinguished. Below 57°C, unfolding was rate-determining whereas above that temperature aggregation was rate-determining.

Functional properties of BSA

Foaming

Foaming may be defined as the creation and stabilization of gas bubbles in a liquid. Proteins diffuse to the air-water interface and reduce surface tension. At the interface they partially unfold and associate to produce an intermolecular cohesive film with some degree of elasticity. Foam expansion and stability improved when BSA interacted with basic proteins like lysozyme and clupeine (Poole *et al.*, 1984) due to cross-links formed between BSA and lysozyme at the interface. BSA on its own performs best near its isoelectric point when electrostatic repulsion is at its minimum. When it interacts with lysozyme, the greatest expansion and stability was found between pH 8 and 9, which is between the isoelectric point of BSA (4.7) and lysozyme (10.7) when the proteins are oppositely charged. Clupeine with a pI at 12 was found to be more effective than lysozyme. Lipids inhibit foaming by displacing protein molecules from the air-liquid interface and by disrupting the integrity of the protein film (Ross, 1950). This lipid inhibition was counteracted when BSA interacted with clupeine at the air-liquid interface (Poole *et al.*, 1986).

Gelation properties of BSA

BSA when heated formed soluble aggregates through disulphide and noncovalent bonds. Alpha-Lactalbumin, on the other hand does not form soluble aggregates on its own but interacts with BSA through disulphide interchange to form soluble aggregates (Matsudomi *et al.*, 1993). Soluble aggregates of polymerized molecules were formed during the early stages of heat-induced gelation of proteins, and subsequent polymerization resulted in the formation of a rigid gel network (Nakamura *et al.*, 1986; Kitabatake *et al.*, 1989). The addition of alpha-lactalbumin to BSA reduced the gelling ability of BSA, as measured by the complex modulus G* (Paulsson *et al.*, 1986).

Gelation according to Ferry (1948) is a two step mechanism. An initiation step involving unfolding or

dissociation of the protein molecules, followed by an aggregation step in which association or aggregation reactions occur, resulting in gel formation under appropriate conditions. For the formation of a highly ordered gel, it is essential that the aggregation step proceed at a slower rate than the unfolding step (Hermansson, 1978; 1979a). The denaturation temperature of BSA by differential thermal analysis (DTA) was found to be 64°C (Itoh *et al*., 1976) and 62°C by differential scanning calorimetry (Ruegg *et al*., 1977). The denaturation temperature of BSA increased when it binds to fatty acids (Bernal and Jelen, 1985) but reduced when it interacts with clupeine (Poole *et al*., 1987). Therefore it appears that the interaction of one biopolymer eg. fatty acid to BSA led to stability of the BSA molecule, whereas another molecule eg. clupeine interacted and facilitated the initiation of unfolding of BSA.

Ligand-binding

Perhaps, the most outstanding property of albumin is its ability to bind reversibly an incredible variety of ligands (Goodman, 1958; Daughaday, 1959; Yates and Urguhart, 1962; Jacobsen, 1969; Klopfenstein, 1969; Burke *et al*., 1971; Unger, 1972; Westphal and Harding, 1973; Beaven *et al*., 1974; Jacobsen, 1977; Richardson *et al*., 1977; Spector and Fletcher, 1978; Brodersen, 1979; Adams and Berman, 1980; Savu *et al*., 1981; Roda *et al*., 1982). BSA is the principal carrier of fatty acids that are otherwise insoluble in circulating plasma. It also performs many other functions such as, sequestering oxygen free radicals and inactivating various toxic lipophilic metabolites such as bilirubin (Emerson, 1989). Albumin has a high affinity for fatty acids, hematin, bilirubin and a broad affinity for small negatively charged aromatic compounds. It forms covalent adducts with pyridoxyl phosphate, cysteine, glutathione, and various metals, such as Cu (II), Ni (II), Hg (II), Ag (II), and Au (I). As a multifunctional transport protein, albumin is the key carrier or reservoir of nitric oxide, which has been implicated in a number of important physiological processes, including neurotransmission (Stamler *et al*., 1992). It also belongs to a multigene family of proteins that include alpha-fetoprotein (AFP) and vitamin D-binding protein (VDP), which is also known as G complement (GC) protein. Although AFP is considered the fetal counterpart of albumin, its binding properties are distinct and it has been suggested that AFP may have higher affinity for some unknown ligands important for fetal development. VDP plays an important role in calcium regulation. ADP and VDP both interact with the class II major histocompatibility complex suggesting that these proteins may play an important role in modulating the immune system (van Oers *et al*., 1989). In circulating plasma approximately 30% of free sulphydryl, Cys-34, is oxidized by cysteine and glutathione (Peters, 1985).

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